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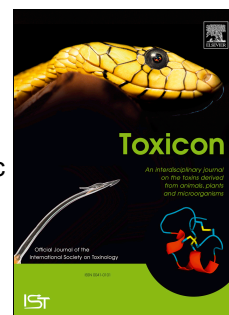
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**Characterising the enzymatic profile of crude tentacle extracts from the
South Atlantic jellyfish *Olindias sambaquiensis* (Cnidaria: Hydrozoa)**

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23 Highlights

- 24 Biologically active toxins were obtained from tentacle extracts in good yields
- 25 Metalloproteinase, serine proteinase and phospholipase A₂ activities were detected
- 26 The data validated previously predicted toxins in *O. sambaquiensis* proteome
- 27 Activity of serine proteinase and phospholipase A₂ were comparable to viper venoms
- 28 Presence of these enzymes may explain some symptoms of human envenomings

Abstract

Jellyfish venoms are of medical and biotechnological importance, with toxins displaying antimicrobial, analgesic and anti-tumor activities. Although proteolytic enzymes have also been described, detailed characterisation of these proteins is scant in *Olindias spp.* High throughput mass spectrometry profiling of cnidarian venoms has become increasingly popular since the first description of the proteomic profile of putative toxins isolated from nematocysts of the hydrozoan jellyfish *Olindias sambaquiensis* describing the presence of orthologous enzymes as presented in venoms of advanced species as snakes. Rigorous bioinformatics analyses can aid functional annotation, but biochemical assays are prerequisite to unambiguously assign toxic function to a peptide or protein. Here we present results that experimentally confirm previously predicted proteomic analysis that crude venom extracts from tentacles of *O. sambaquiensis* are composed of polypeptides with metalloproteinase, serine proteinase and phospholipases A₂ activities. Surprisingly, levels of serine proteinase and phospholipase A₂ activities were comparable to those observed in venoms of *Bothrops* snakes which were used as positive controls in this study. Hence, these data offer new opportunities to explore serine proteinase and phospholipase A₂ activities in the clinical sequelae following *O. sambaquiensis* envenomation, with future possible biopharmaceutical applications.

Keywords: cnidaria, venom, metalloproteinase, serine proteinase, phospholipase A₂, *Olindias sambaquiensis*

Introduction

Human encounters with jellyfishes are common, although envenomation vary in toxicity ranging from mild symptoms to sometimes lethal consequences depending on several factors including the species or geographical distribution (Haddad Junior et al. 2002; Lumley et al. 1988). Different toxic effects have been reported in jellyfish extracts such as neurotoxic (Carneiro et al. 2011; Lassen et al. 2012), myotoxic (Endean 1987; Yanagihara and Shohet 2012) and pore forming cytolytic biological activities (Haddad Junior et al. 2014; Li et al. 2013; Long and Burnett 1989; Wang et al. 2013). Enzymes such as proteases (Calton and Burnett 1982; Calton and Burnett 1983; Gusmani et al. 1997; Lee et al. 2011) and phospholipases (Carneiro et al. 2011; Gusmani et al. 1997; Stillway and Lane 1971) have also been reported in venoms of some jellyfish species, as have toxins with potential biotechnological interest including antimicrobial peptides from *Aurelia aurita* (Shenkarev et al. 2012), toxins with antineoplastic and analgesics effects from *Pelagia noctiluca* (Ayed et al. 2012a; Ayed et al. 2012b).

Olindias sambaquiensis (Müller, 1861) is a hydrozoan jellyfish species endemic to the southwestern Atlantic, and is fairly common from the north coast of the State of São Paulo (Brazil) to the province of Buenos Aires (Argentina) (Oliveira et al. 2016; Vannucci 1951). The species has already been associated with accidents with swimmers and fishermen, in which the victim developed an intense painful condition and a skin pattern of the lesions of short round marks, or sometimes with short welt lines, the lesions may develop into blisters or necrosis (Haddad Junior et al. 2010; Mosovich and Young 2012; Resgalla Junior et al. 2011). Several reports are available on *O. sambaquiensis* envenomings. However, biochemical characterisation of the toxic components of *O. sambaquiensis* venom is scarce.

The first use of high throughput mass spectrometry to characterise a jellyfish venom described the putative toxins isolated from nematocysts of *O. sambaquiensis* (Weston et al. 2013). This study notably also revealed that amino acid sequences of these putative toxins were orthologs to many representatives of peptide toxins found in higher animals, strongly implicating that venoms may have arisen from basal metazoan origins. The putative toxins of *O. sambaquiensis* were dominated by

proteases and phospholipases A₂ mostly from snake venoms. Proteases are abundant toxins especially in the venom of Viperidae snakes, with important biological roles leading to hemostatic disturbances, cytotoxicity and tissue disruption (Markland 1998; Moura-da-Silva et al. 2007). Phospholipase A₂ are also a major components of snake venoms with potentially life-threatening neurotoxic and myotoxic action (Gutiérrez and Lomonte 2013). Modern molecular biology techniques such as genomics, transcriptomics and proteomics can generate huge amounts of sequence data relatively quickly. This has certainly resulted in an explosion of new toxins being described in known and newly described venomous animals. Advanced bioinformatics methods can be applied to functional annotation (Gacesa et al. 2015; Starcevic et al. 2015), but biological assays are required to unambiguously assign toxin function. In this study, we present the results of an analysis that aimed to experimentally confirm the presence of enzyme activities previously identified by mass spectrometry proteomics of venom isolated from nematocysts of *O. sambaquiensis*, and to determine function by comparison with the activities of similar enzymes from snake venoms.

Experimental

1. Collection of animals and preparation of crude tentacle extracts

An expedition to collect specimens of hydromedusae *O. sambaquiensis* was held on September 23th 2014 in the São Sebastião Channel, northern coast of the State of São Paulo, Brazil, along the Enseada and Cigarras beaches (23°41'28.5"S 45°21'25.6"W) at an average depth of 10 m. Animals were caught using trawling nets with a 25 mm mesh. Each trawl lasted approximately 20 mins. Specimens of *O. sambaquiensis* were morphologically identified based on the presence of four radial canals of orange color with pleated gonads along these canals, long marginal tentacles present in large numbers and bell diameter not exceeding 10 cm (Vannucci 1951). Animals were transported alive in buckets with local sea water to the Centro de Biologia Marinha (CEBIMar), Universidade de São Paulo in São Sebastião, where tentacles were excised and stored in microtubes with seawater on dry ice for transport to the Laboratório de Imunopatologia, Instituto Butantan where the samples were stored at -80 °C until required. The tentacles were centrifuged for 3 mins at 3,000 x g at 4 °C to remove sea water and the tentacles then gently homogenized in a tissue homogeniser in 2 mL 10 mM sodium acetate, pH 5.0. The homogenate was then centrifuged for 5 mins at 15,000 x g at 4 °C and the supernatant recovered. The protein concentration of the crude tentacle extract was determined using a Bradford assay (Bradford 1976) and the electrophoretic profile was analysed by SDS-PAGE (Laemmli 1970).

2. Proteolytic activity assays

Metalloproteinase activity of *O. sambaquiensis* tentacle extracts was assessed by Fluorescence Resonance Energy Transfer (FRET) using the following substrates: Abz-AGLA-EDDnp (GenOne, Rio de Janeiro, RJ, BR), with broad specificity; Mca-PLAQAV-Dpa-RSSSR-NH₂ (R&D Systems, Minneapolis, MN, USA), specific for ADAMs (A Disintegrin And Metalloproteinases) and Mca-PLGL-Dpa-AR-NH₂ (R&D Systems, Minneapolis, MN, USA), specific for MMPs (Matrix Metalloproteinases) and the results compared to the metalloproteinase activity of *Bothrops jararaca* crude venom, run at the same experiments, as a control of an animal venom in which metalloproteinase activity is relevant. For the assays, Abz-AGLA-EDDnp was made up to a concentration

of 200 μM in 10 mM CaCl_2 , 150 mM NaCl and 0.05 % (v/v) Brij 35 (dissolved in 50 mM sodium acetate pH 5.0 or 50 mM Tris to give two solutions at pH 7.5 or 9.0), as described by Kuniyoshi *et al.* (2012), with modifications. The reaction mixture contained 50 μL containing 10 μg tentacle extract or 1 μg *B. jararaca* venom and 50 μL of substrate which were incubated at 4 $^\circ\text{C}$, 25 $^\circ\text{C}$ or 37 $^\circ\text{C}$ for 10 mins. Jararhagin (5 $\mu\text{g}/\text{mL}$), a *B. jararaca* venom metalloproteinase (Paine et al. 1992), was used as positive control. The reactions were monitored every minute over the 10 mins reaction using a SpectraMax M2 fluorimeter (Molecular Devices, Sunnyvale, CA, USA) at λ_{EM} 420 nm and λ_{EX} 320 nm in kinetic mode. The specific activity of three independent experiments was calculated in relative fluorescence units (RFU/min/ μg). Mca-PLAQAV-Dpa-RSSSR- NH_2 was dissolved to 10 μM in 25 mM Tris containing 2.5 mM of ZnCl_2 and 0.005 % Brij 35, pH 9.0. The reaction mixture contained 50 μL substrate and 50 μL containing 10 μg tentacle extract or 1 μg *B. jararaca* venom. Recombinant enzyme ADAM-17 (R&D Systems, Minneapolis, MN, USA) was used as a positive control at a concentration of 0.2 $\mu\text{g}/\text{mL}$. Mca-PLGL-Dpa-AR- NH_2 was used at a concentration of 10 μM in buffer 50 mM Tris containing 10 mM CaCl_2 , 150 mM NaCl and 0.05 % Brij 35, pH 7.4. Again, the reaction mixture contained 50 μL substrate and 50 μL containing 10 μg tentacle extract or 10 μg *B. jararaca* venom. Recombinant enzymes MMP-2 and MMP-9 also used at 0.2 $\mu\text{g}/\text{mL}$ concentrations as positive controls. All reaction mixtures were monitored every minute using a SpectraMax M2 fluorimeter (Molecular Devices, Sunnyvale, CA, USA) at λ_{EM} 405 nm λ_{EX} 320 nm in kinetic mode. The specific activities of MMPs and ADAMs were calculated from three independent experiments in U/mg using the conversion factor (pmol/RFU) derived from the standard curve of 7-amino-4-Methylcoumarin (Sigma-Aldrich, St. Louis, MO, USA).

Serine proteinase activity of *O. sambaquiensis* tentacle extracts was assessed by the method of Zhu *et al.* (2005) and the results also compared to the serine proteinase activity of *B. jararaca* crude venom, run at the same experiments, as a control of an animal venom in which serine proteinase activity is relevant. The assays were performed using 20 μL of 500 μM of the chromogenic substrate L-benzoyl-arginyl-p-nitroanilide (L-BAPNA) (Sigma-Aldrich, St. Louis, MO, USA). The substrate was incubated at 4 $^\circ\text{C}$, 25 $^\circ\text{C}$ or 37 $^\circ\text{C}$ for 40 mins with 20 μL containing 5 μg tentacle extract

or 5 μ g *B. jararaca* venom and 160 μ L 50 mM sodium acetate buffer, pH 5.0 or 50 mM Tris-HCl pH 7.5 or 9.0. As a positive control, porcine pancreas trypsin (Sigma-Aldrich, St. Louis, MO, USA) was used at a concentration of 25 μ g/mL. Proteolysis of the substrate was measured at 405 nm.

3. Zymography

In-gel zymography (Heussen and Dowdle 1980; Snoek-van Beurden and Von den Hoff 2005; Vandooren et al. 2013) was used to estimate the molecular mass of proteases in the tentacle extract using casein and gelatin as indicator substrates. Samples (20 μ g) of tentacle extract were separated by SDS-PAGE using either 12.5 % (w/v) polyacrylamide gel containing 2 mg/mL casein or 15 % (w/v) polyacrylamide gel containing 2 mg/mL gelatin. Gels were washed twice for 20 min with 2.5 % (v/v) Triton X-100 to remove SDS and to renature the proteins. The gels were then incubated for 17 hours at 37 °C in 0.5 mM CaCl_2 , 20 mM Tris pH 7.4 to allow enzymatic digestion of the substrates. The gels were then visualized by staining with 0.125 % (w/v) Coomassie Blue R-250 in 45 % (v/v) methanol and 10 % (v/v) acetic acid for 1 hr and then destained with 40 % (v/v) ethanol and 10 % (v/v) acetic acid until the hydrolysis bands became visible.

4. Fibrinolytic activity

The fibrinolytic activity of *O. sambaquiensis* extract was determined on fibrin plates similar to the technique described to snake venom tests by Baldo et al. (2008). In a petri dish, we mixed 10 mL of warm solution 2 % Agarose Low Melting (Amresco[®], Solon, OH, USA) with 10 mL of 3 mg/mL fibrinogen type I from bovine plasma (Sigma-Aldrich, St. Louis, MO, USA), diluted in 0.05 M Tris-HCl, 0.2 M NaCl, 0.05 M CaCl_2 , pH 7.3 plus in the same buffer, 1 mL of 1 M CaCl_2 and added 4 μ L of thrombin (10 U/ μ L) obtained from bovine plasma (Sigma-Aldrich, St. Louis, MO, USA). The plates were kept at room temperature for about 2 hours to solidify the agarose. After this period, six wells were molded in the fibrin plate and 40 μ L of each sample containing 20 μ g, 30 μ g and 40 μ g crude tentacle extracts were placed in their respective well in the plate. As a positive controls jararhagin and *B. jararaca* venom were used at a concentration of 10 μ g/well (previously standardized) and as a negative control we used 40 μ L of 10 mM Tris buffer. The plates were incubated at 37 °C for 17 hours and the fibrinolytic activity

was calculated from the area (mm²) of the hydrolysis halos of three independent experiments.

5. Phospholipase A₂ activity

The presence of phospholipase A₂ activity of *O. sambaquiensis* tentacle extracts was determined using a modification of the method described by Fernández Culma *et al.* (2014) and the results compared to the phospholipase A₂ activity of *B. jararaca* and *Bothrops jararacussu* crude venoms, run at the same experiments, as controls of animal venoms in which phospholipase A₂ activity is moderate and relevant, respectively. Briefly, 20 µL containing 5 µg tentacle extract or viperid venoms were incubated with 20 µL of 320 µM 4-Nitro-3-[octanoyloxy] benzoic acid (Enzo Life Sciences, Farmingdale, New York, USA) in 200 µL of 10 mM sodium acetate buffer, pH 5.0; or 10 mM Tris, pH 7.5 or 9.0, containing 10 mM CaCl₂, 100 mM NaCl. The reaction mixtures were incubated for 40 mins at 4 °C, 25 °C or 37 °C and hydrolysis determined at 425 nm. All experiments were performed in triplicate.

Results

1. Crude venom extraction

A total of 40 *O. sambaquiensis* hydromedusae specimens with a bell diameter ranging from 4-8 cm were collected after 6 trawls. Initial attempt to extract crude venom from isolated nematocysts (Weston et al. 2013) did not yield sufficient active protein for the enzyme assays. However, homogenization of tentacles in low osmolality buffer recovered 2-6 mg/mL of predominantly with high molecular mass soluble proteins (Figure 1), sufficient for conducting the assays.

2. Proteolytic activity using peptide substrates

In order to confirm and quantify the presence of activity of the main proteolytic enzymes described in the proteome (Weston et al. 2013), tentacle extracts of *O. sambaquiensis* were assayed against broad substrates for metalloproteinases and serine proteinases using a range of different pH and temperature reaction conditions. The maximum proteolytic activities against all peptide substrates were detected using buffer at pH 7.5 and a reaction temperature of 37 °C (Figure 2). Then, the extract was tested against selected substrates for metalloproteinases (MMPs and ADAMs). The crude tentacle extract of *O. sambaquiensis* showed proteolytic activities against all tested substrates, confirming previous proteomic evidence for the presence of these enzymes (Table 1). However, proteolytic activity was low against substrates for metalloproteinases, whereas serine proteinase activities were high and comparable to the levels of activity detected from *B. jararaca* venom, in which serine proteinases are abundant and very important constituents for the coagulant disrupting properties of the venom.

3. Proteolytic activity using macromolecular substrates

Zymography was used to detect the presence of proteolytic enzymes in tentacle extracts of *O. sambaquiensis* by in-gel digestion of casein and gelatin. Strong bands of casein hydrolysis corresponded to proteins with molecular masses of approximately 36 kDa and 30 kDa, two weaker hydrolytic bands between 60 and 45 kDa (Figure 3A). Strong bands of gelatin hydrolysis corresponded to proteins with molecular masses of 50 kDa, 24 kDa and 20 kDa (Figure 3B).

Olindias sambaquiensis tentacular extract was also tested for fibrinolytic activity on agarose plates containing fibrin. Control samples of jararhagin and *B. jararaca* venom showed fibrinolytic activity in all tests, whereas *O. sambaquiensis* extracts (20, 30 and 40 µg/well) did not induce fibrin hydrolysis in any of the experiments (data not shown).

4. Phospholipase activity

Activity of phospholipase A₂, another important protein detected in *O. sambaquiensis*' proteome, tested positive at different temperatures and pHs, with the highest activity achieved in tests using pH 7.5 and 37 °C (Figure 4A). Comparisons of extract activity with activity of viperid venoms indicated that extract showed phospholipase activity higher than the one observed in *B. jararaca* venom and about half of the activity presented by *B. jararacussu* venom (Figure 4B), known as one with the highest phospholipase A₂ activity amongst *Bothrops* snakes.

Discussion

Marine animal venoms have received special attention in recent years, as the realization that toxins of marine origin may provide an as yet relatively untapped reservoir of new leads for pharmaceutical discovery (Ayed et al. 2012a; Ayed et al. 2012b; Shenkarev et al. 2012; Ver Donck et al. 2014; Zaharenko et al. 2011). The recent application of mass spectrometry for high throughput proteomics has facilitated the rapid characterization of toxins from many marine and terrestrial venomous animals. However, annotation of peptide sequences by comparison to even specialised toxin databases (e.g. the UniProtKB/Swiss-Prot Tox-Prot program, (Jungo et al. 2012)) is not sufficient to also imply functional homology i.e. that a peptide that has a toxic physiological role in one animal will also have toxic activity in another. Although advanced bioinformatics techniques can help discriminate peptides with toxin functions from those peptides with other non-toxic physiological activities (Gacesa et al. 2015; Starcevic et al. 2015), ultimately only biochemical and physiological assays are sufficient to unambiguously assign a toxin function.

Studying cnidarian venoms for function using biological assays has some limitations, mainly because the toxins are not secreted from venom glands, like in snakes, but are secreted by specialised nematocyst organelles that are present all over the animals. This makes the obtaining pure venom technically challenging, time consuming and usually results in poor yield (Yanagihara and Shohet 2012). In this study, a Percoll-sucrose density gradient centrifugation method was used to first separate the nematocysts from the tentacle material (Weber et al. 1987), and then isolated nematocysts were sonicated aiming to disrupt the membrane and consequently release the proteins, as performed by (Weston et al. 2013). However, although this method provided sufficient material for high throughput proteomics analysis of *O. sambaquiensis* (Weston et al. 2013), it failed to provide sufficient amounts of protein to proceed with the enzymatic assays. A new approach was applied, targeting to stimulate the release of nematocyst's toxins by extrusion through tentacle homogenization (Endean and Henderson 1969; Tamkun and Hessinger 1981) in distilled water (Ayed et al. 2012a; Ayed et al. 2012b; Gusmani et al. 1997; Hessinger and Lenhoff 1973). However, the osmotic methodology, by using distilled water, was

not successful, resulting in denatured proteins of obtained as a precipitate. We adapted previous methods performing the extraction under low salt solution (10 mM sodium acetate, pH 5.0). This approach resulted in significant protein yields with toxins displaying preserved activity, which could subsequently be measured in the various enzymatic assays employed.

The presence of proteases and phospholipase A₂ in the crude tentacle extracts was detected using specific synthetic substrates under different reaction conditions of pH and temperature. The presence of these enzymes in *O. sambaquiensis* extracts were predicted previously (Weston et al. 2013). Interestingly, most of the enzymes predicted by the referred proteomic data belongs to high animals, specifically snakes. Thus, in this study we compared the activity of proteases and phospholipases A₂ present on jellyfish tentacle extracts and viper snake venoms.

Concerning proteases, tentacle extracts of *O. sambaquiensis* were active against the serine proteinase substrate L-benzoyl-arginyl-p-nitroanilide, at levels close to the ones obtained with *B. jararaca* venom. This observation supports the presence of enzymes recognized as serine proteinases from *B. jararaca* and *Crotalus adamanteus* venoms predicted by Weston and collaborators (2013). Interestingly, the most prominent enzymes predicted by the jellyfish proteome were metalloproteinases recognized as viper venoms' class P-I SVMs, but metalloproteinase activity was very low in the tentacle extracts. One explanation for low levels of metalloproteinase activity could be substrate specificity since the assays were carried out using synthetic small peptides. However, our data was based in three different substrates: a broad-specific metalloproteinase substrate and two others, selective for MMPs and ADAMs, respectively, suggesting that metalloproteinase activity do not correlate with the prominent identification in the proteome. Nevertheless, using complex substrates as gelatin and casein, bands of approximately 25 and 50 kDa, which correspond to the molecular masses of snake venom metalloproteinases were detected besides bands of approximately 30 kDa, consistent to serine proteinases molecular mass.

Proteases play an important role in pathologies, especially following envenomation by Viperid snakes (Moura-da-Silva et al. 2007). Catalytic actions of SVMs can be correlated to major biological effects such as induction of hemorrhage,

apoptosis of endothelial cells and pro-inflammatory action (Moura-da-Silva et al. 2007). The fact that kinetic assays using tentacle extract of *O. sambaquiensis* gave lower metalloproteinase activity supports the absence of bleeding in recreational bathers following contact with *O. sambaquiensis* (Haddad Junior et al. 2002; Haddad Junior et al. 2010; Mosovich and Young 2012; Resgalla Junior et al. 2011), which might suggest metalloproteinases play a minor role in the envenomation frame, despite being the most abundant in the proteome.

The action of serine proteinases in snake venoms is mostly directed to several components of coagulation cascade, leading to a typical and frequent consumption syndrome in patients bitten by *Bothrops* snakes presenting unclotable blood when admitted to the hospital care (Serrano and Maroun 2005). Such symptoms have never been reported in human cases of *O. sambaquiensis* envenomation, which typically present as severe inflammation (Haddad Junior et al. 2002; Haddad Junior et al. 2010; Kokelj et al. 1993; Mianzan et al. 2001; Mosovich and Young 2012; Resgalla Junior et al. 2011). Serine proteinases also act on substrates that form the complement cascade, leading to the activation of pro-inflammatory mediators that could explain part of the symptoms observed in jellyfish patients (Sim and Tsiftoglou 2004). A difference in substrate specificity between *O. sambaquiensis* and *Bothrops* serine proteinases might explain the differences in symptoms following envenomation by these two animals, which deserves further investigation if serine proteinases were to be pursued as possible pharmacological leads.

Different forms of phospholipases A_2 have also been predicted by *O. sambaquiensis* proteome analysis, resembling toxins from scorpions, elapid and viperid snakes (Weston et al. 2013) and this prediction was confirmed in our assays. Crude tentacle extracts of *O. sambaquiensis* were found to have approximately half of phospholipase activity showed by *B. jararacussu* venom, in which phospholipase A_2 is the most abundant toxin (Sousa et al. 2013), and much higher activity than *B. jararaca* venom. Our data was consistent with previous reports comparing phospholipase activity of tentacle extracts from *Phyllorhiza punctata* jellyfish to that of *Crotalus durissus terrificus* snake venom (Carneiro et al. 2011). Phospholipases A_2 are found at

high concentrations and are a highly toxic component of the venoms of many diverse animals (Harris 1985). They display a broad spectrum of action, including haematological and immune system dysfunction, neurotoxicity, myotoxicity and notable an acute edematogenic response (Kini and Evans 1989). The presence of phospholipase A₂ activity in jellyfish venom, evidencing the importance of these enzymes for cnidarians was already reported (Gusmani et al. 1997; Helmholz et al. 2007; Hessinger and Lenhoff 1973; Stillway and Lane 1971). Nevalainen et al. (2004) measured the phospholipase A₂ activity in the tissue homogenates of 22 species of Cnidarian, showing its role in envenomation by members of this phylum. Furthermore, phospholipase A₂ has previously been described in the venoms of two scyphozoan jellyfish species, *Cyanea capillata* and *Cyanea lamarckii* and, have been attributed with inflammatory reactions at the site of envenomation (Helmholz et al. 2007). Over 80 % of human cases present with gross, localized edematogenic lesions following envenomation by *O. sambaquiensis* (Haddad Junior et al. 2010). This suggests that phospholipases A₂ might be the main enzyme toxin responsible for the clinical presentation of edema in humans following an *O. sambaquiensis* sting, especially since haemorrhagic lesions typical of serine proteinases have not reported.

Conclusions

A new method to obtain crude tentacle extracts with retained biological activity of *O. sambaquiensis* toxins is described which could now be applied to other cnidarian species, thus providing a new biochemical tool with which to screen basal metazoan venoms for pharmacologically active peptides. Biological data on the selected enzymes studied here validate previous annotation of peptide sequences assembled using a mass spectrometry platform for high throughput proteomics (Weston et al., 2013). Comparing the *in vitro* activity of the crude venom extract presented here, with symptoms observed following human contact with *O. sambaquiensis*, we suggest that at least serine proteases and phospholipases A₂ may be involved in the clinical sequelae of *O. sambaquiensis* envenomation.

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Legends for Figures

Figure 1 – Electrophoresis profiles of crude *Olindias sambaquiensis* tentacle extracts.

Samples (15 µg) were separated by one dimensional SDS-PAGE using 12.5 % (w/v) polyacrylamide under non-reducing (A) or reducing (B) conditions. Protein bands were stained using Coomassie Blue R-250.

Figure 2 – Proteolytic activity at different conditions of pH and temperatures.

For metalloproteinae assay (A), extracts (10 µg) were incubated with 200 µM of Abz-A-G-L-A-EDDnp substrate and hydrolysis was monitored on fluorimeter at λ_{EM} 420 nm and λ_{EX} 320 nm. Results are expressed as relative fluorescence units (RFU/min/µg). For serine proteinase activity (B), 5 µg extracts were incubated with 500 µM serine proteinase substrate L-BAPNA for 40 min. Proteolysis of the substrate was measured at 405 nm and expressed as absorbance units (ABS/min/mg). The results correspond to the mean \pm sd of three independent experiments.

Figure 3 – Zymography of *Olindias sambaquiensis* tentacles extract. Extracts (20 µg)

were submitted under non-reducing condition to SDS-PAGE 12.5 % containing casein (A) or SDS-PAGE 15 % containing gelatin (B) as substrate. The gels were incubated for 17 hours at 37 °C with 20 mM Tris containing 0.5 mM CaCl₂, pH 7.4, allowing enzymatic digestion of substrates. The gels were stained with Coomassie Blue R-250. Arrows indicate hydrolysis bands.

Figure 4 – Phospholipase A₂ activity of *Olindias sambaquiensis* tentacles extract.

Extracts (5 µg) were incubated with 320 µM of substrate in a range of different pH and temperature reaction conditions, with hydrolysis measured at 425 nm (A). Comparison of phospholipase A₂ activity of *O. sambaquiensis* tentacles extracts and snake venom (*B. jararaca* and *B. jararacussu*). Activity was measured at 425 nm after incubation at 37 °C in buffer pH 7.5. Results are expressed in absorbance units (ABS/min/mg) as the mean \pm sd of three independent experiments (B).

Table 1: Proteolytic activity of crude *O. sambaquiensis* tentacle extracts against specific substrates.

Enzyme	Substrate	Specific activity
Metalloproteinase	Broad (Abz-AGLA-EDDnp)	BJ venom: 227.46 ± 39.43 RFU/min/μg OS extract: 1.95 ± 0.19 RFU/min/μg
	MMP (Mca-P-L-G-L-Dpa-A-R-NH ₂)	BJ venom: 0.05 ± 0.01 U/mg OS extract: 0.02 ± 0.00U/mg
	ADAM (Mca-P-L-A-Q-A-V-Dpa-R-S-S-S-R-NH ₂)	BJ venom: 0.48 ± 0.00 U/mg OS extract: 0.02 ± 0.00 U/mg
Serinoproteinase	Benzoyl-Arginyl-p-nitroanilide	BJ venom: 0.49 ± 0.04 ABS/min/mg OS extract: 0.37 ± 0.02 ABS/min/mg

The results are expressed as a mean of three independent experiments ± standard deviation.

RFU - Relative Fluorescence Units; ABS – Absorbance Units at 405 nm; BJ – *B. jararaca*, OS – *O. sambaquiensis*, MMP – Matrix Metalloprotease; ADAM – A Disintegrin And Metalloprotease.

